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Note

Separation of collagen types I and III by high-performance column liquid chromatography

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Two main principles have been applied in the past to this category of separations; namely, gel permeation chromatography and ion-exchange chromatography on cellulose-based ion exchangers [1]. In each case the so-called soft sorbents were used which do not offer the possibility of using high pressures and the separation therefore takes several days. The number of theoretical plates is low and the efficiency of the separation is also low. Recently, the so-called rigid sorbents have been introduced into the separation of proteins, allowing the application of small diameter particles and consequently elution under high pressure [2]. Sorbents in this category offer the possibility of applying the principles of gel permeation chromatography, ion-exchange chromatography and reversed-phase high-performance liquid chromatography (HPLC). The first successful separations were done by making use of controlled-pore glass: more recent are those derived from silica gel or various copolymers. In these sorbents the surface is covered with various forms of hydroxylated compounds. Of these so-called glycophases, those like LiChrosorb-Diol [3], TSK-SW gels [4] and Separon HEMA 1000 Glc [5] should be mentioned. These gels meet the demands of HPLC and offer separations of about two orders more rapid in comparison to the classical ones.

Recently a system of chromatographic procedures (multidimensional chromatography) was described for the separation of different types of collagen and constituent α-chains [6]. This system, though fairly efficient, exploits classical liquid column chromatographic procedures: two successive DEAE-cellulose chromatographic steps and two successive separations on Bio-Gel A 1.5 m. The success of this procedure is based on the preliminary removal

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of proteoglycans during the first DEAE-cellulose run. The identification of collagen types I and III* in this system is based on the presence of the disulphide bonds in collagen type III; as these bonds are absent in collagen type I, the limited proteolysis of insoluble collagen type I results in two types of α-polypeptide chains [α_1(I) and α_2, molecular weight 100,000]. However, limited proteolysis of type III collagen, due to the disulphide bonds, results in the formation of [α_1(III)]$_3$ trimer molecules (molecular weight 300,000). Instead of Bio-Gel separation, chromatography on Sepharose CL-6B of these two collagen types was suggested recently by ChandraRajan and Klein [8]. In addition, it is also possible to obtain fair separations of collagen types I and III by CM-cellulose chromatography (Epstein [9], Chung and Miller [10]), where [α_1(III)]$_3$ molecules exhibit an intermediate mobility between α_1(I) and α_2 molecules.

The main disadvantages of all methods described up to now are: (1) the impossibility of distinguishing between $(\alpha_1)_2\alpha_2$ and [α_1(III)]$_3$ molecules; (2) long separation time; and (3) difficulties in separating individual α-chains [α_1(I), α_1(III)] side by side.

In the present study we have used Separon HEMA 1000 Glc (a copolymer of 2-hydroxyethyl methacrylate with ethylene dimethacrylate covalently coated with glucose), because it has been found in preliminary experiments that this sorbent separates the compounds not only according to molecular size but under specified conditions it offers the possibility of separating compounds of equal molecular mass as well.

EXPERIMENTAL

Chromatographic separation

This was done using a Pye-Unicam liquid column chromatograph LC 20 equipped with the UV detector LC-3. For detection the wavelength was set at 230 nm. A stainless-steel column [11], 500 × 8 mm, packed with Separon HEMA 1000 Glc (12—17 μm; Laboratory Instrument Works, Prague, Czechoslovakia) was used [12]. The apparatus was operated at a flow-rate of 1.5 ml/min and 1.5 MPa over-pressure. The sensitivity of the detector was set at 0.04, the recorder speed was 0.25 cm/min. The whole separation lasted less than 30 min. Elution was carried out isocratically with 0.05 *M* Tris · HCl (pH 7.5) that was 2 *M* with respect to urea.

Pepsin solubilization of collagens

The method used follows almost completely the procedure described by ChandraRajan and Klein [8]. Skin (1 g) from two-month-old rat was finely cut with scissors, extracted overnight with chloroform—methanol (2:1, v/v) and then with methanol for 6 h at 4°C. The defatted tissue was suspended in 0.5 *M* acetic acid (10 mg/ml). Then crystalline pepsin (Worthington Biochemical Corp. Freehold, NJ, U.S.A.) was added at a concentration of 1 mg/ml [5] and the reaction mixture was stirred for 24 h at 8°C. The digest was then

*Since the nomenclature of collagen types and polypeptide changes that constitute the tropocollagen triple helix is rather complex, the reader not familiar with this is referred to ref. 7.

centrifuged at 30,000 *g* for 1 h at 4°C. The insoluble residue was redigested overnight with additional pepsin (the same enzyme:substrate ratio) and centrifuged as above. Supernatants were pooled and dialyzed overnight against 0.02 *M* Na_2HPO_4. The resulting collagen precipitate was collected by centrifugation at 30,000 *g* within 1 h. A flow scheme for this preparation is given in Fig. 1.

Denaturation of pepsin-solubilized collagen

The collagen precipitate was suspended (2—10 mg/ml) in, or dialyzed against, 2 *M* urea containing 0.05 *M* Tris · HCl (pH 7.5) and denatured by heating to 45°C for 30 min. When necessary the material loaded on the column

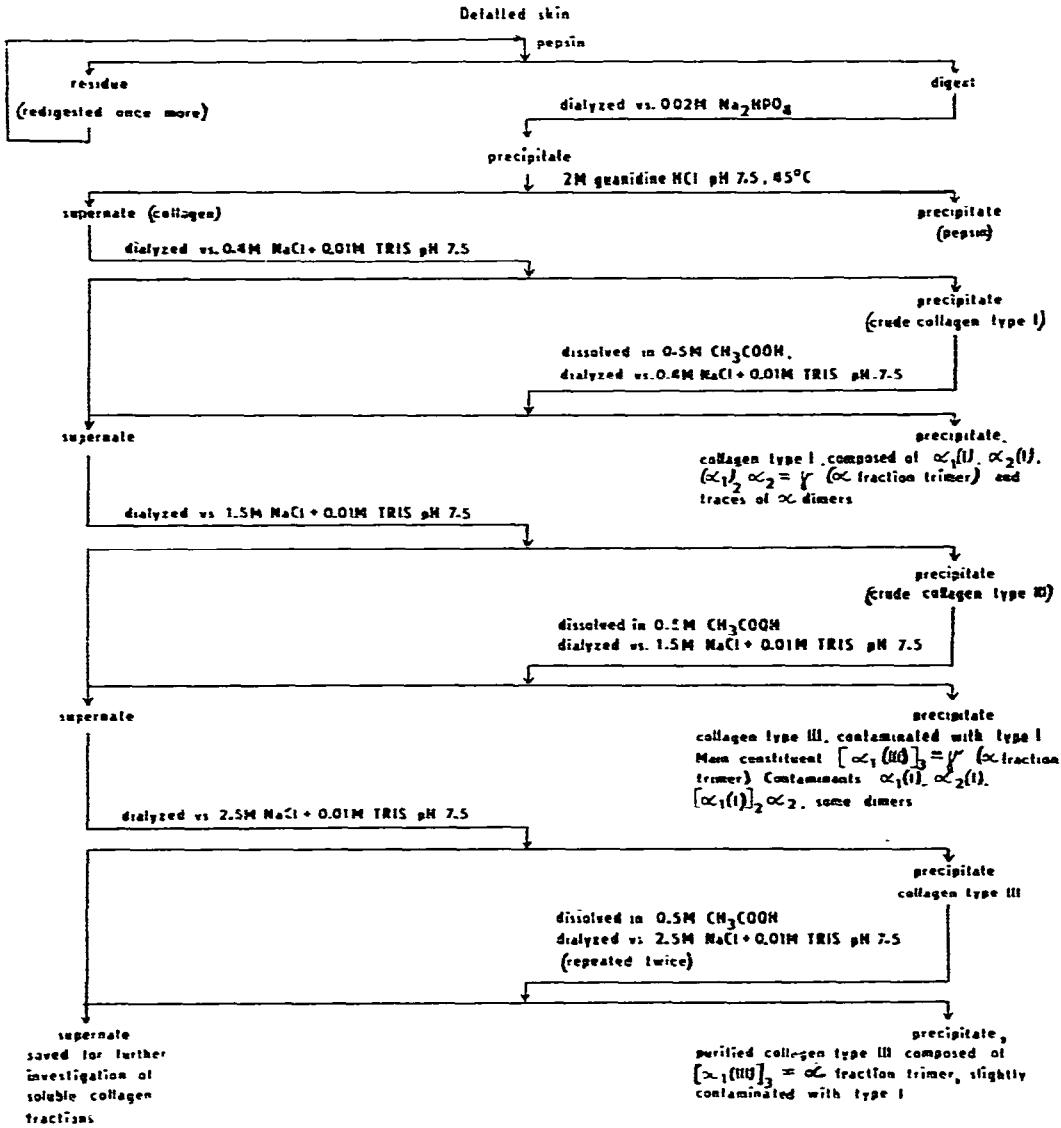

Fig. 1. Flow scheme for preparation of individual collagen types.

was concentrated by adding Aquacide IIA (flake polyethyleneglycol) B grade (Calbiochem, San Diego, CA, U.S.A.) and by removing the gel by brief centrifugation.

Cleavage of disulphide bonds

In order to avoid problems resulting from high UV absorbancy of mercaptoethanol in UV light, S—S bonds were cleaved by performic acid [12]. Denatured collagen was mixed (equal volumes) with concentrated formic acid, and hydrogen peroxide (30%) was added to a final concentration of 2%. The reaction mixture was left for 2 h at room temperature and then loaded onto the column.

Note: Individual collagen fractions isolated by column chromatography and fraction precipitation were checked by their amino acid composition, behaviour during SDS-slab gel electrophoresis [13] and CM-cellulose or Bio-Gel A 1.5 m chromatography [6]. Only those preparations that withstood the above criteria were used for high-resolution liquid column separations.

RESULTS AND DISCUSSION

The applicability of the separation procedure is illustrated in Figs. 2—4. As would be expected, preparations of type I collagen yielded α_1 and α_2 fractions with some β and γ material and slightly contaminated with some breakdown products (Fig. 2). Type III collagen preparations indicated the presence of $[\alpha_1(III)]_3$ which, in spite of the most cautious way of preparation, was contaminated with $\alpha_1(I)$ and $\alpha_2(I)$ material as well as with some other trimer. This material was indistinguishable both chromatographically and electro-

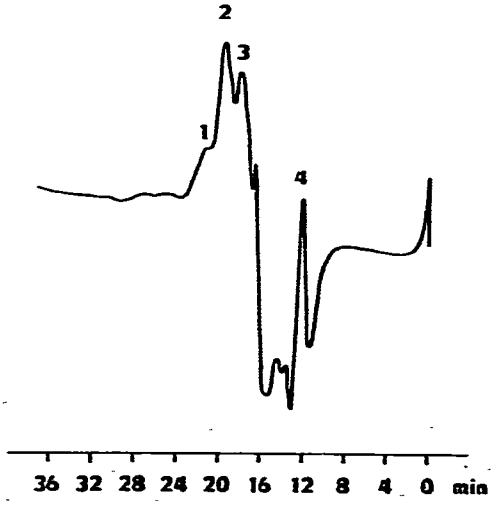

Fig. 2. Separation of polypeptide chains present in collagen type I preparation (10 µl injected). Peaks of intermediate mobility between α and γ are (according to polyacrylamide gel electrophoresis) β fractions (dimers of non-specified α-chains). 1 = Degradation products; 2 = $\alpha_1(I)$; 3 = $\alpha_2(I)$; 4 = γ = $[\alpha_1(I)]_2\alpha_2$.

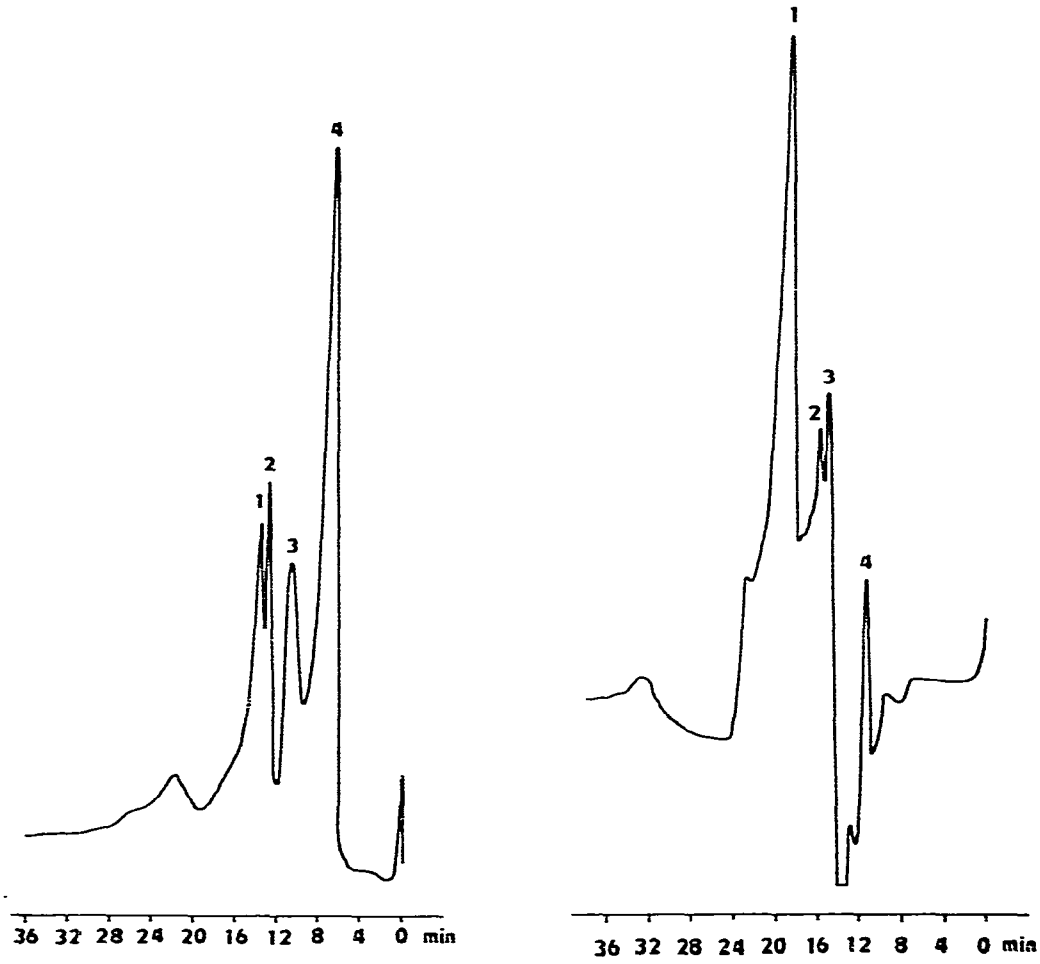

Fig. 3. Separation of polypeptide chains present in collagen type III preparation (2 μl injected). 1 = α_1(I); 2 = α_2(I); 3 = $[\alpha_1(I)]_2\alpha_2$; 4 = γ = $[\alpha_1(III)]_3$.

Fig. 4. Separation of polypeptide chains present in collagen type III preparation after performic acid treatment (2 μl injected). 1 = α_1(III); 2 = α_1(I); 3 = α_2(I); 4 = $[\alpha_1(I)]_2\alpha_2$.

phoretically from $(\alpha_1)_2\alpha_2$ trimer (Fig. 3). After performic acid treatment a pronounced peak of α_1(III) appeared with a mobility lower than both alphas of type I collagen (Fig. 4). Also in mixtures of type I and type III collagen it was possible to distinguish between all three types of α-chains.

Similarly, differences in mobility were also seen in trimers, i.e. native type I collagen. $(\alpha_1)_2\alpha_2$ could be distinguished from type III collagen molecule $[\alpha_1(III)]_3$. For unknown reasons, however, the mobility of $[\alpha_1(III)]_3$ is the highest of all peaks seen while $[\alpha_1(I)]_2\alpha_2$ is the peak to follow. This is just the opposite order of retention to that observed with single α-chains where the order of elution was α_2, α_1(I), α_1(III). Similar relations within dimers (β-chains) were not investigated as these were virtually absent in our pepsin-treated insoluble collagen.

It also appears worth mentioning that in all situations type III collagen preparations were heavily contaminated with collagen type I chains while preparations of collagen type I were devoid of substantial contamination with type III collagen. It was also observed that the contaminating collagen type I α-chains in type III collagen were never in the expected $\alpha_1 : \alpha_2$ 2:1 ratio. Instead, a higher proportion of α_2-chains was consistently seen (Fig. 3). Whether this reflects a higher affinity of α_2-chains for the $[\alpha_1(III)]_3$ molecule or whether this reflects some alternative in assembling individual α-chains into collagen molecules is certainly difficult to decide. The first alternative seems more plausible as long as the other explanation does not fit the generally accepted image about the α-chain composition of individual collagen types.

Obviously, several mechanisms rule this separation. One of these is the molecular-sieve effect that categorizes time periods in which trimers, dimers and eventually monomeric α-chains elute from the column. This mechanism is, however, not the only one as separation of two categories of trimers — $[\alpha_1(I)]_2\alpha_2$ and $[\alpha_1(III)]_3$ — was achieved as well as the separation of three different α-chains. Possible reasons which can be accounted for in separations of molecules with equal molecular weights can be summarized as follows:

(1) Different molecular shapes would lead to different hydrodynamic volumes of separated molecules and thus the elution in three, more or less resolved, α-chain peaks in gel permeation chromatography will result even if there is no other mechanism operative. Calibration of the Separon HEMA Glc column with linear polydextran standards (Pharmacia, Uppsala, Sweden) in 0.05 *M* Tris · HCl + 2 *M* urea shows that this is the main separating mechanism.

(2) Adsorption or partition interactions may be contributing to the resolution obtained by gel permeation chromatography. Hydrophobic adsorption properties of the non-modified Separon HEMA matrix [14] were largely suppressed by the covalent coating [5] with glucose in the sorbent Separon HEMA Glc used in this work. However, some remaining adsorption of hydrophobic domains of collagen α-chains in the eluent used cannot be completely disregarded.

(3) Possible weak selective affinity of collagen chains to the glucose-coated macroporous adsorbent causing respective retention differences of otherwise similar molecules.

It appears that the gel permeation chromatography is primarily controlling the separation; the other mechanisms mentioned most probably contributed to better resolution in this particular case. A detailed study of the role of these interaction principles in fast and highly efficient chromatographic separations of collagen polypeptide chains is being carried out.

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